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Identification of cytotoxin-producing *Klebsiella oxytoca* strains isolated from clinical samples with cell culture assays

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ABSTRACT

Background: *Klebsiella oxytoca* is an opportunistic pathogen which damages intestinal epithelium through producing cytotoxin tilivalline. This toxin plays a role in the pathogenesis of bacteria and is the main virulence factor which leads to antibiotic-associated hemorrhagic colitis progress.
Materials and methods: In this study, we collected a total of 75 *K. oxytoca* strains isolated from the stool, urine, blood, wounds, and sputum and evaluated them in terms of the production of toxins; we detected their cytotoxic effects on HEp-2 cells.
Results: Of all the isolates, five *K. oxytoca* strains isolated from the stool cultures, two strains isolated from the blood cultures, one strains isolated from the wound cultures, and one strains isolated from the urine cultures had cytotoxic effects on HEp-2 cells. The strains isolated from sputum cultures had no cytotoxic effects on HEp-2 cells.
Conclusions: In the current study, the majority of strains isolated from the stool of the patients included cytopathic effects on HEp-2 cells.

1. Introduction

K. oxytoca is a member of the Enterobacteriaceae family. This bacterium is non-motile, Gram-negative, rod-shaped and is considered as an opportunistic pathogen. Currently, *K. oxytoca* is considered as a clinically significant pathogen which is associated with infections in hospitalized patients, including children, newborns, and individual with immune deficiency. *K. oxytoca* as a remarkable opportunistic pathogen can play an important role for the generation of various infections acquired in a hospital and community. This bacterium is usually isolated from patients with Septicemia, bacteremia, septic arthritis, soft-tissue infections, cholecystitis, urinary tract infections, and most recently it has been also isolated from babies with colic [1].

Studies have shown that *K. oxytoca* can demonstrate its pathogenesis via producing cytotoxin in human. *K. oxytoca* strains associated with the colitis and mucosal skin infections in humans are able to produce cytotoxin, which may be somewhat due to the pathogenesis of this bacterium [2]. The feature of this cytotoxin was introduced in 1989 and 1992, when it observed that this bacterium could round and kill the cell lines of HEp-2, HeLa, CHO, and Vero in vitro [3,4]. In addition, as it was reported, this cytotoxin was sensitive to heat, resistant to digestion by proteinase, had low molecular weight, and its molecular formula was C8H15O4N3 [5]. Recently, this non-protein cytotoxin has been also called Tilivalline (TLV) which is a member of pyrrolobenzodiazepine (PBD) family. It is a nonribosomal peptide enterotoxin and is introduced as a new class of cytotoxic intestinal bacteria [6]. In addition to *K. oxytoca* strains, *Micrococcus* and *Streptomyces* strains are also able to produce Tilivalline cytotoxin [7]. Tilivalline is the only pyrrolobenzodiazepine (PBD) that is produced in the microbiota of human gut and can disturb the intestinal epithelial barrier via the induction of

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apoptosis and reduction of epithelial resistance [6]; as a result, PBDs are known as altered metabolites of acinetobacter DNA [8]. *K. oxytoca* is recognized as one of the major causes of antibiotic-associated hemorrhagic colitis (AAHC) in adolescents and adults [9,10]. Researchers have recently discovered the how colitis is caused by molecular mechanisms of *K. oxytoca*. Cytotoxin is one of the important pathogenic factors, which plays a role in the virulence of this bacterium. Cytotoxin is associated with the pathogenesis of *K. oxytoca* [10,11]. The cytotoxic effects on epithelial cells of the human are not observed in other strains of *Klebsiella* spp and are only observed in *K. oxytoca* strains. This study aimed to identify toxin-producing *K. oxytoca* strains isolated from clinical samples by HEp-2 cell culture.

2. Materials and methods

2.1. Bcterial strains

A total of 75 strains of *K. oxytoca* were collected from several hospitals in Tehran from 2015 to 2016. *K. oxytoca* strains were isolated from 3750 clinical samples, including stool, blood, urine, sputum, and wounds. In this study, a strain of *K. oxytoca* was also isolated from the stool culture of a patient with diarrhea who was suspected to *Clostridium difficile*.

To identify the strains of *K. oxytoca*, we used morphology of colonies on MacConkey agar (Merck, German), and the standard microbiological tests. In addition, we used PCR to verify the isolates of *K. oxytoca* by identification of *pehx* gene.

2.2. Verification of K. oxytoca isolates

All bacterial strains which underwent standard microbiological tests in microbiology laboratories and were detected as *K. oxytoca* strains were also detected and verified by PCR through amplification of Polygalacturonase specific gene (*pehX*).

2.3. Molecular detection of pehX gene

Genomic DNA of *K. oxytoca* isolates was obtained through boiling two or three colonies in 500 μl of distilled water for 10 min which were then centrifuged for 10 min at 10000 rpm. The supernatants was used as the template for amplification. To identify *pehX* gene, forward primer PEH C and reverse primer PEH D, with the following sequences, were used: forward primer PEH C (5 ′GAT ACG GAG TAT GCC TTT ACG GTG -3′), reverse primer PEH D (5′- TAG CCT TTA TCA AGC GGA TAC TTG -3 ′) [11,12]. PCR reaction was performed in a final volume of 20 μl. To perform each PCR reaction, we used 10 μL master mix (Ampliqon, Denmark), 0. 5 μl forward Primer 10 pmol (Bioneer, Daejeon, Korea), 0. 5 μl reverse Primer 10 pmol (Bioneer, Daejeon, Korea), 8. 5 μl distilled water, and 50 ng of bacterial DNA.

PCR reaction was performed in a thermocycler (PEQLAB, Germany) with an initial denaturation at 95 °C for 5 min; and 35 cycles including denaturation steps at 94 °C for 45 s, the annealing at 52 °C for 45 s, the extention at 72 °C for 45 s, and final extention at 72 °C for 10 min. The electrophoresis of PCR product was performed in a 1% agarose gel. The gel was stained using ethidium bromide (50 mg/L), and it was detected by Gel Doc (GVM20 model syngene).

2.4. 4. Cytotoxin tissue culture assay

The cytotoxicity assays of *K. oxytoca* strains were performed using previously described methods [10,13]. In brief, HEP-2 cell line (ATCC CCL-23) was used for screening this cytotoxin. A 1:1 dilution of the filtered supernatant from culture of *K. oxytoca* strains with PBS was added to the each well of a 96-well plate was seeded with 1×10^5 HEp-2 cells and inoculated onto HEp-2 cells followed by incubation in 5% carbon dioxide at 37° C for 72 h. A positive cytotoxic effect was recorded as cell rounding under light microscopy. The positive control of Cytotoxin-Producing *K. oxytoca* MH43-1 was a gift from Dr Christoph Hoegenauer, Department of Internal Medicine, Medical University of Graz, Austria. The *K. oxytoca* strain ATCC 13182 served as negative control.

3. Results

A total of 75 isolates of *K. oxytoca*, 11 (14.7%) of isolates recovered from blood, 2 (2.7%) of isolates recovered from wounds, 4 (5.3%) of isolates recovered from respiratory cultures, 51 (68%) of isolates recovered from urine cultures and 7 (9.3%) of isolates recovered from stool cultures.

In this study, all *K. oxytoca* strains isolated from stool cultures of patients with diarrhea, and from urine, blood, wounds, and sputum cultures were evaluated in terms of the production of cytotoxin by HEp-2 cell culture. Of all the isolates, five *K. oxytoca* strains isolated from the stool cultures, two strains isolated from the blood cultures, one strain isolated from the wound cultures, and one strain isolated from the urine cultures had cytotoxic effects on HEp-2 cells (Fig. 1). The strains isolated from sputum cultures had no cytotoxic effects on HEp-2 cells. The toxin-negative *K. oxytoca* ATCC13182 supernatants had no effects on HEp-2 cell cultures (Fig. 2).

4. Discussion

K. oxytoca is a commensal bacterium existing in the intestinal flora; it is present in the intestines of 2%–10% of healthy people [9]. However, this bacterium is introduced as an opportunistic pathogen, and nowadays it is considered as a clinically significant pathogen [1].

Cytotoxin is one of the important pathogenic factors, which plays an important role in the virulence of this bacterium and is associated with pathogenic effects of *K. oxytoca* [14].

The cytotoxic effects on epithelial cells of human are not observed in other strains of *Klebsiella* spp and are only observed in *K. oxytoca* strain. A part of the pathogenic effects of the bacteria is attributed to the production of cytotoxin. The capacity of *K. oxytoca* strains to produce toxins is a major factor in the pathogenesis of

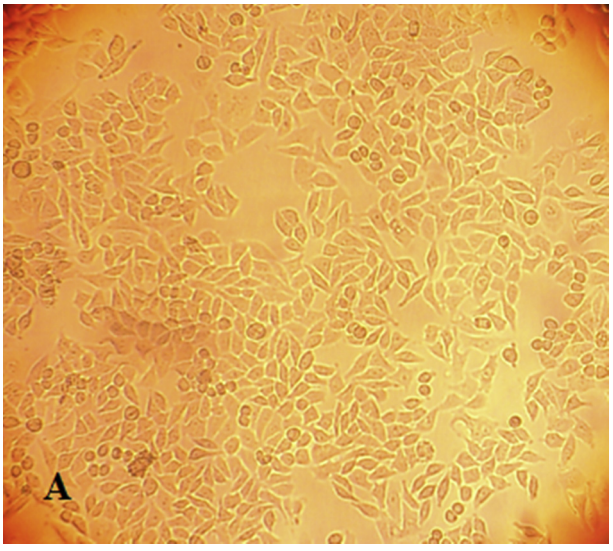

Fig. 1. Toxin negative *K. oxytoca* ATCC13182 supernatants on HEp-2 cell cultures.

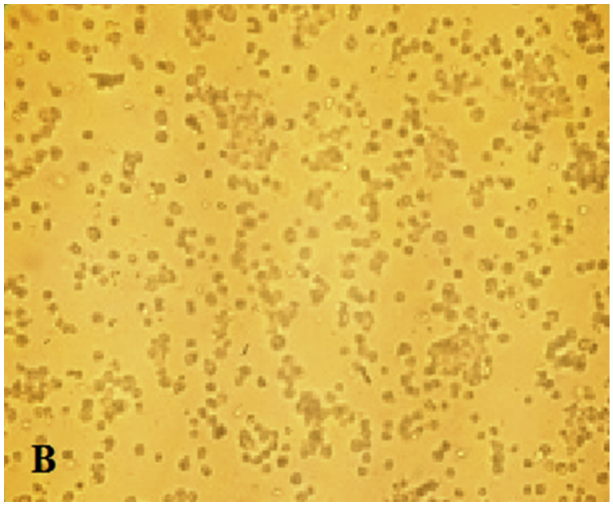

Fig. 2. Cytotoxic effects of *K. oxytoca* supernatants strain isolated from patient on HEp-2 cells.

AAHC [6]. In addition, the *K. oxytoca* has shown pathogenic effects on laboratory animals such as mice, rat, rabbit, voles, and mole. Darby et al. studied the cytotoxic and pathogenic characteristics of *K. oxytoca* strains isolated from laboratory animals, including rats, non-human primates, pigs, and guinea pig; the results of their study, indicated that tilivalline was the cytotoxin present in these strains [1].

Some studies in other countries have been conducted on toxigenic strains of *K. oxytoca*; according to their results, the prevalence of toxigenic strains is reported between 23% and 82%. In this study of 75 strains of *K. oxytoca*, nine strains (12%) were toxigenic. The high prevalence of toxigenic strains of *K. oxytoca* in some studies might be because the studied strains were isolated only from patients with antibiotic-associated hemorrhagic colitis (AAHC). Since, cytotoxin is a major virulence factor which is involved in AAHC progress and is associated with AAHC pathogenesis, the number of toxigenic strains of *K. oxytoca* isolated from patients' samples is usually higher. In this study, we used cell culture to study the toxigenic effects of *K. oxytoca* strains isolated from urinary tract infection, bacteremia, respiratory tract infection, diarrhea, colitis, abscess, and wounds.

In this study, *K. oxytoca* strains isolated from stool cultures of patients with diarrhea, and from urine, blood, wounds, and sputum cultures were inestigate in terms of the production of cytotoxin by HEp-2 cell culture. Of all the isolates, *K. oxytoca* strains isolated from the stool, blood, wound, and urine cultures had cytotoxic effects on HEp-2 cells. The samples isolated from sputum cultures had no cytotoxic effects. The majority of studies conducted in other countries have assessed the toxigenic effects of *K. oxytoca* strains isolated from patients with AAHC.

In a study by Joining et al., which was conducted in Austria in 2009, a total of 13 strains of *K. oxytoca* isolated from stool cultures of healthy subjects, 10 strains isolated from urine cultures, 12 strains isolated from blood cultures, 16 strains isolated from wound, 15 strains isolated from patients with AAHC, and 14 strains isolated from stool cultures of patients with diarrhea were assessed in terms of cytotoxic effects on HEp-2 cells. Of all, six strains of *K. oxytoca* isolated from stool cultures of healthy individuals, two strains isolated from blood cultures, six strains isolated from wound cultures, nine strains isolated from stool cultures of patients with AAHC, and nine strains isolated from stool cultures of patients with diarrhea had cytotoxic effects on HEp-2 cells. In strains isolated from urine and sputum samples, no cytotoxic effect was observed. According to the results of this study, the incidence of colitis was attributed to the production of toxins, which was the result of enormous growth of *K. oxytoca* strains isolated from patients and healthy subjects undergoing antibiotic treatment [2].

Hogenauer et al., conducted a study on 22 patients with Antibiotic-Associated Colitis (AAC) in Austria in 2006. Of the 22 patients, six patients were diagnosed with AAHC. *K. oxytoca* strains were isolated from the stool cultures of five patients with AAHC; all these isolates produced cytotoxin and had a cytotoxic effect on HEp-2 cells [15].

In a study by Hoffmann et al., in Austria in 2010, *K. oxytoca* was isolated from the stool culture of a 15-year-old male patient with acute urinary infection who was affected by bloody diarrhea after treating with amoxicillin and clavulanate. The *K. oxytoca* strain produced cytotoxin and had cytotoxic effects on HEp-2 cells [11].

Ulbi et al., conducted a study in Australia in 2012 and evaluated 20 patients with AAHC diagnosed between 1996 and 2008. After carrying out an assay for cytotoxic activity on the HEp-2 cells, cytotoxic effects on HEp-2 cells were observed in 6 isolates of *K. oxytoca*, which was due to the production of cytotoxin [16].

In a study by Tsang LL et al., which was conducted in Hong Kong in 2011, a total of 6488 stool and rectal swab samples were collected from 4170 patients with diarrhea who were hospitalized in the general ward and bone marrow transplantation ward from October 2009 to November 2010. A total of 120 *K. oxytoca* isolates was identified. Of all, 36 isolates of *K. oxytoca* (30%) were capable of producing cytotoxin and had cytotoxic effects on HEp-2 cells. The findings of this study showed that toxin-producing strains of *K. oxytoca* were resistant to ampicillin and cephalothin [17].

In a study by Kathrin A. et al., which was conducted in Austria in 2013, of 74 strains of *K. oxytoca* collected in Germany, Spain, United States of America, Hong Kong, Japan, Netherlands, and Austria, 39 isolates produced cytotoxin and had cytotoxic effects on HEp-2 cells. Most of the toxin-producing strains were isolated from stool samples of patients with AAHC, diarrhea, while a small number of the strains were isolated from patients with colitis and asymptomatic carriers [9].

Ines Zollner-Schwetz et al., conducted a study on 235 patients with inflammatory bowel diseases (including 150 patients with Crohn's disease and 85 patients with ulcerative colitis). They isolated 11 strains of *K. oxytoca*. According to their findings, 2 isolates produced tilivalline toxins on HEp-2 cells and rounded the cells and showed cytopathic effects. In addition, of 12 asymptomatic intestinal carriers, 4 isolates were toxigenic [7].

In a study by Alikhani. et al., which was conducted in Hamadan, Iran from May 2011 to Dec 2013 faecal samples were collected from hospitalized patients receiving antibiotic treatment. The clinical isolates was cultured on Hep-2 cells for cytotoxin production. Out of 331 samples collected from patients, 40 were confirmed molecularly to be clinical isolates of *K. oxytoca*. 40 isolated strains showed cytotoxin activity on Hep-2 cells [18].

In most studies, *K. oxytoca* strains isolated from patients with antibiotic-associated hemorrhagic colitis (AAHC) have been assessed in terms of toxigenicity; in addition, these studies have evaluated the role of these bacteria in the incidence of gastrointestinal infections. However, the use of clinical specimens other than the stool is less studied. In this study, in addition to examining the role of these bacteria in the incidence of gastrointestinal, role of these bacteria in the incidence of urinary, respiratory, skin, and bacteremia infections have evaluated, the *K. oxytoca* isolates were also assessed in terms of toxigenicity. In our study, similar to other studies, the majority of strains with cytopathic effects on HEp-2 cells were isolated from the stool of the studied patients. In conclusion, the majority of strains with cytopathic effects on HEp-

2 cells were isolated from the stool of the studied patients; as seen in similar studies.

Conflict of interest

The authors declare that there was no conflict of interest.

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